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13. ABSTRACT (Maximum 200 Words)

Murine models of prostate cancer have been developed that rely on the somatic activation of β-catenin. The approach employs Cre-loxP mediated targeted genetic recombination of the $Catnb^{+/lox(ex3)}$ locus. Expression of Cre was targeted specifically to the prostate secretory epithelium using androgen responsive minimal probasin (PB) or prostate specific antigen (PSA) gene promoters. Lesions produced by PB-Cre were limited in comparison to the previously reported MMTV-Cre mice, while PSA-Cre caused highly inflammed and invasive lesions. In contrast to the MMTV-Cre, no squamous metaplasia was produced by the PB- or PSA-Cre. No lesions were detected with K14-Cre mice, that target expression of Cre to basal cells. These observations are likely to be related to the type of cells or stages of differentiation targeted by each promoter. The ontogeny of PINs and carcinomas are being traced, using simultaneous Cre dependent expression of lacZ. In a limited screen of clinical histology samples, stabilization of β-catenin in PIN lesions was readily detected in some but not all PIN lesions. NKCC1 was generally not extinguished in human PINs, as well as in murine prostate cancers caused by genetic alterations other than β-catenin (SV40-T, or active AKT). Potential cross talk of the β-catenin and PI3kinase signaling pathways, and downstream gene targets in prostate cancer are under investigation.

14. SUBJECT TERMS
β-catenin, prostatic intra epithelial neoplasia, PTEN, myc, cyclin D1, Cre/loxP.

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Initiating events in prostate cancer: The role of somatic activation of β-catenin.

Introduction.

The genetic basis of prostate cancer is just now beginning to unravel, with the characterization of chromosomal losses frequently associated with the cancer, and identification of a number of candidate gene, such as PTEN, MXI1, RB, p53, NKX3.1 that have been implicated in the progression of the disease, based on chromosomal analysis as well as animal modeling. Little is known about the mechanisms of initiation of prostate cancer.

Histopathological studies of prostate cancers have led to the identification of a specific type of lesion that represents the primary precursor of human prostate cancer, known as prostate intra epithelial neoplasia (PIN) (1). PIN is recognized as a continuum between low-grade and high-grade forms, with high-grade PIN most likely representing the immediate precursor of early invasive carcinoma. Characteristic architectural and cytological features are shared between PIN lesions and early invasive carcinomas including multifocal nature of the lesions, and common chromosomal abnormalities (reviewed in (2)). We have reported characteristic appearance of PINs upon stabilization of β-catenin in the prostate (3). Stabilization was achieved through Cre/loxP mediated excision of the third exon of β-catenin. This model was based on the MMTV-LTR driven expression of Cre in a wide range of secretory epithelia, and skin. Interestingly, other tissues affected underwent squamous metaplasia, rather than neoplastic transformation. In the prostate, the lesions were bening, with no sign of invasion or infiltration, but with limited squamous metaplasia. The lack of association of the PINs with progression to malignancy was assumed to reflect a specific role for β-catenin in the initiation of prostate cancer. Initiation of benign adenomatous polyps in the bowels is a well established role for β-catenin, and these, while non-invasive, are considered to be the immediate precursors of invasive carcinoma and colon cancer (4, 5). The research underway aims to clarify the role of β-catenin in prostate cancer, its relevance to human prostate cancer, and the cross talk of this signaling pathway with other events associated with human prostate cancer.

Body.

Task 1.

To establish the role of β-catenin in the initiation of prostatic intraepithelial neoplasia (PIN) like lesions.

Cre mediated site directed mutagenesis of endogenous target genes has been used as a general strategy for inducing prostate cancer. By this strategy we are able to activate defined oncogenes, e.g. stabilize β-catenin, while simultaneously inactivating tumor suppressor genes, e.g. PTEN, or promoting expression of marker genes, such as lacZ or influenza HA.

In the course of the past year we have tested a number of potentially useful mouse models for controlled prostate specific expression of Cre. Initially we examined the possibility of tetracycline (tet) dependent expression of Cre, using double transgenic MMTV-tTA x tet-O-Cre mice. To this aim we obtained mice from the laboratory of Lothar Hennighausen, that expressed a tetracycline sensitive tet gene transactivator (tTA) under the control of the MMTV-LTR (6). We also obtained from the laboratory of Jeff Gordon mice that expressed Cre in a tTA dependent manner. The tTA activates upstream tet operator sequences linked to a minimal promoter from human cytomegalovirus (tetO- P(hCMV)-Cre) (7). Compound mutant MMTV-tTA x tet-O-Cre x $Catnb^{+/lox(ex3)}$ mice were generated and weaned in the absence of tetracycline, so as to allow the expression of Cre. Preliminary observations suggested that a fraction of (less than 1/3) these mice were indeed susceptible to PIN lesions, similar to those of the MMTV-Cre x $Catnb^{+/lox(ex3)}$ mice (3). However, the prostatic lesions were less severe, and the unpredictable nature of their occurrence, the leakiness of their regulation, as well as extra prostatic manifestations caused by the wide spread expression of Cre, and the general poor health of the mice complicated their further use.

As an alternative approach we have attempted to generate mice that express CreERT2 under the control of the prostate specific Probasin promoter (8). The aim was to flank the CreERT2 cDNA with loxP sequences, and insert a reporter gene (influenza HA) downstream of this cassette, such that Cre activity self excises the encoding gene and allows the expression of the reporter gene. A schematic representation of this construct is shown in Figure 1. The construction of this "tamoxifen dependent" self inactivating cassette proved difficult, primarily because of frequent excision of the CreERT2 during amplification of the DNA in bacteria, and the instability of the construct.

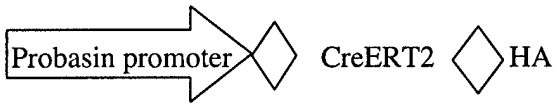

Figure 1. Schematic representation of a tamoxifen inducible self inactivating Cre cassette. Self-excision of CreERT2 allows expression of the linked HA reporter gene.

As a third approach we obtained through a collaboration with Dr. Valera Vasiokhin transgenic mice that express Cre under the control of the minimal probasin (PB) promoter (9), or the prostate specific antigen (PSA) promoter. The PSA-Cre mice were described before (10, 11). The PB-Cre mice are not published, and have been generated and characterized by Valera Vasioukhin. Both promoters are androgen dependent.

These mice were crossed with Catnb$^{+/lox(ex3)}$ mice, and male progeny of 3 months of age were analyzed for prostate lesions. So far all double transgenic male progenies examined have PIN or more progressed prostate cancer in some or all the prostate lobes (Figure 2). The PB-Cre mice generate less aggressive and more limited disease, in comparison with the PSA-Cre mice, which appear to have even more advanced cancer than the MMTV-Cre mice. Unlike the MMTV-Cre or MMTV-tTA mice, the PB-Cre or PSA-Cre mice do not appear to generate lesions in other tissues, and subsequently the mice are otherwise in good health. Surprisingly, the lesions caused by PSA-Cre appear to be massively infiltrated with inflammatory cells. There may be also invasion of the stroma and of

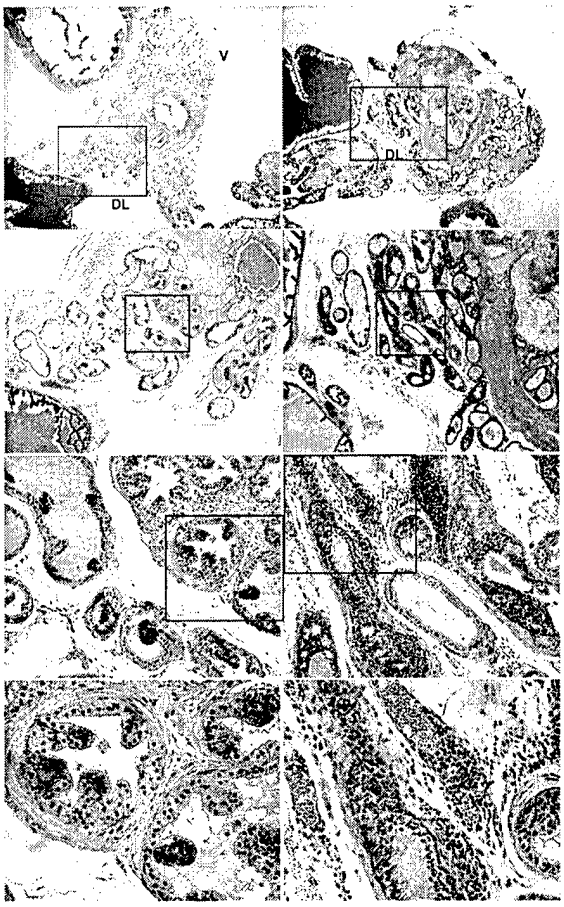

Figure 2. Prostatic lesions observed in PB-Cre x Catnb$^{+/lox(ex3)}$ mice (left column), or PSA-Cre Catnb$^{+/lox(ex3)}$ mice (right column). Descending panels show increasing magnifications, with red squares designating the areas that are magnified. Note within tumor areas nuclear polymorphism, hyperchromia, and prominent nucleoli. In comparison, the neighboring healthy secretory cells form a regular monolayer, with underlying elongated basal cells. Lesions obtained with PSA-Cre mice were significantly more chaotic, posssibly invasive, and appeared to be infiltrated with large numbers of inflammatory cells. DL: dorsolateral prostate, V: ventral prostate.

blood vessels by tumor cells, but the presence of large numbers of tumor infiltrating cells make it difficult to be confident of the extent of progression of the tumors, and more careful analysis is needed.

Task 2.

To test the hypothesis that basal cells or a basal cell related stem cell is the target of oncogenic action of β-catenin.

Mice expressing CreERT2 under the control of the keratin 14 promoter were obtained from the laboratory of Dr. Pierre Chambon. These mice had been reported to express a tightly tamoxifen regulated Cre activity in their skin (12). We expected Cre activity in the prostates of these mice to be limited to the basal cells. The keratin 14-CreERT2 mice were crossed to $Catnb^{+/lox(ex3)}$ mice and the compound mutant progeny were induced by i.p. injection of 4-hydroxytamoxifen. To our surprise the compound mutant mice showed a severe skin phenotype, even in the absence of treatment with tamoxifen. Histologic examination of sections of the skin showed lesions very similar to those previously reported in the MMTV-Cre x $Catnb^{+/lox(ex3)}$ mice: follicular epitheloid cysts, trichofollilculomas containing sebaceous glands, epidermal hyperplasia and aberrant invaginations. Examination of the prostates of these mice did not reveal any PINs. The lack of phenotype in the prostate was later confirmed with a closely related transgenic mouse line, keratin 14-Cre mice, obtained also from Dr. Chambon. These express a constitutively active Cre.

The absence of PIN lesions argues against a precursor-progeny relationship between the prostatic basal cells and the PINs. This notion is supported by occurrence of PIN and progressive prostate cancer in $Catnb^{+/lox(ex3)}$ x Cre double transgenic male mice, where expression of Cre is targeted to the mature prostate secretory epithelium, using the minimal PB or PSA promoters (see previous section).

Our observation of tamoxifen independent activity of Cre in keratin 14-CreERT2 mice contradicts the original report on this transgenic mouse line. One possible explanation may be the high accessibility of the β-catenin locus to Cre mediated recombination. This notion occurred to us by examining triple transgenic mice that expressed, 1) Cre under the control of the TS4 gut specific promoter (7), 2) the $Catnb^{+/lox(ex3)}$ gene (5), and 3) the

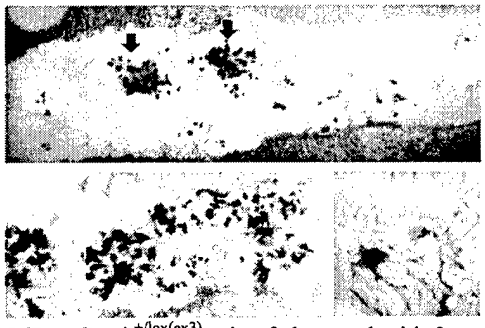

Figure 3. TS4CreERT2 x R26R x $Catnb^{+/lox(ex3)}$ mice fed treated with 2 mg tamoxifen every day for 5 days. Several weeks after the end of the treatment, the mice were sacrificed, the bowels explanted, fixed and stained for β-galactosidase activity, and photographed using a dissection microscope connected to a CCD camera. Note that only fractions of the adenomatous tissue is stained blue, suggesting that recombination of the R26R insert at the ROSA26 locus (that promotes expression of lacZ) is a relatively rare event, as compared with the recombination of the $Catnb^{+/lox(ex3)}$ locus, that is responsible for polyposis. Arrows in the top image point to two "islands" of fused polyps. The image on the lower lefet shows representative regions from another compound mutant mouse. The image on the lower right is of a stained aberrant crypt focus.

R26R allele (13). Cre activity should simultaneously recombine the $Catnb^{+/lox(ex3)}$ locus, inducing polyposis in the bowels, and the R26R locus, allowing expression of lacZ from the endogenous ROSA26 promoter. In this system, the extent to which lacZ activity coincides with polyp formation will dependent on the relative frequencies of Cre mediated recombination in the two loci. Only a fraction of polyps generated in these mice expressed lacZ, suggesting a significantly greater susceptibility for recombination of the β-catenin as compared to the ROSA26 locus (Figure 3). Thus, one can postulate that keratin 14-CreERT2 mice exhibit a low level of Cre activity even in the absence of hormone induction, which is sufficient to recombine the highly accessible β-catenin locus, leaving less accessible sites untouched. This could explain the apparent contradiction between our observations and those reported earlier by the Chambon laboratory.

We are currently crossing the PB-Cre x $Catnb^{+/lox(ex3)}$ as well as the PSA-Cre x $Catnb^{+/lox(ex3)}$ compound mutant mice with R26R mice. Based on our experience with these as well as other Cre transgenic mice, we anticipate in triple transgenic mice to obtain, 1) chimeric expression and activity of Cre, 2) higher frequency of recombination in the β-catenin than R26R locus, resulting in more PINs than lacZ expressing cells. These two factors should allow us to use expression of lacZ as a marker to trace the onngtogeny of the PINs as outlined in the original proposal, and address the potential role of tumor stem cells in the initiation and progression of prostate cancer.

Task 3.

To test the hypothesis that β-catenin induced lesions can progress to carcinomas. The cooperation of PTEN/Akt-1 pathway with β-Catenin.

These experiments are underway.

Task 4.

To investigate the signaling pathways downstream of β-catenin responsible for promoting PIN in the prostate. The requirement for cyclin D1 and c-myc.

These experiments are underway.

Task 5.

To search for evidence implicating β–catenin in human prostate cancer .

We have carried out a limited study of human prostate tumors and PIN tissue sections, made available through our collaboration with M. Loda and S. Signoretti. Paraffin embedded tissue sections were treated for antigen retrieval and stained with antibodies to β-catenin. Among the 11 samples examined, 1 had no visible lesions, 7 contained both invasive and benign lesions, and 4 contained only carcinomas, as summarized in Table 1. Focusing on the benign PIN like lesions, the samples were scored for β-catenin. PINs in two samples stained strongly with the specific antibody, indicating stabilization of β-catenin, while in three other samples the PINs showed a mixed phenotype (Table 1).

patient/sample	carcinoma	PIN	β-catenin in PIN
1	yes	yes	no difference
2	yes	N/A	N/A
3	yes	yes	yes
4	rare	yes	yes
5	yes	N/A	N/A
6	N/A	N/A	N/A
7	yes	rare	partly **
8	yes	N/A	N/A
9	rare	yes	no difference
10	yes	yes	partly
11	yes	yes	partly

Table 1. Stabilization of β-catenin in human prostate cancer. Histologic samples were stained for β-catenin and NKCC1, and scored for presence of invasive lesions (carcinoma), benign lesions (PIN), as well as strong staining for β-catenin (β-catenin in PIN). A vies from the histologic section corresponding to sample 7 (**) is shown in Figure 3.

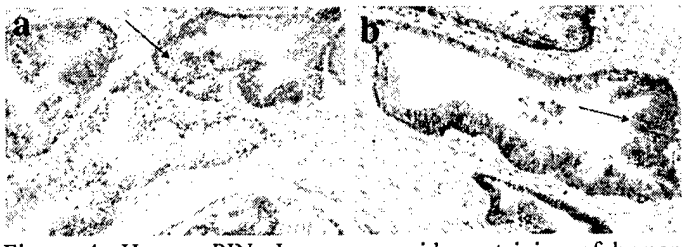

Figure 4. Human PIN. Immunoperoxidase staining of human histology sample 7, as shown in Table 1, for β-catenin. Arrows show the PIN like lesions. A: absence of staining for β-catenin, b: staining for β-catenin.

Figure 4 shows an example of this mixed phenotype, showing both β-catenin postive and negative PINS in sections from the same prostate. Thus, atleast in the sample studies, stabilization of β-catenin could not be exclusively linked with the initiation of prostate cancer. Human PINs are unpredictable in their predisposition to progress to invasive carcinomas, and

this may be related to the initiation of the disease by multiple paths. It remains to be clarified to whether the β-catenin positive lesions are more or less susceptible to progress to malignancy, in comparison to the negative lesions.

We have reported that in the mouse prostate, expression of the Na-K-Cl co-transporter (NKCC1) is restricted to the basolateral region of secretory epithelial cells. Stabilization of β-catenin in the prostate epithelium led to the loss of detectable NKCC1. The NKCC1 (CCC1, BSC2) isoform is present in a wide variety of secretory epithelia and is localized to the basolateral membrane. This raised the possibilities that extinction of this marker may be, a) related to the stabilization of β-catenin, b) related to the loss of epithelial polarization, a common event in most cancers, or 3) restricted to the target cell (prostate secretory epithelium) and its transformation by β-catenin. Experiments were performed to test these possibilities, and to relate our finding to human prostate cancer.

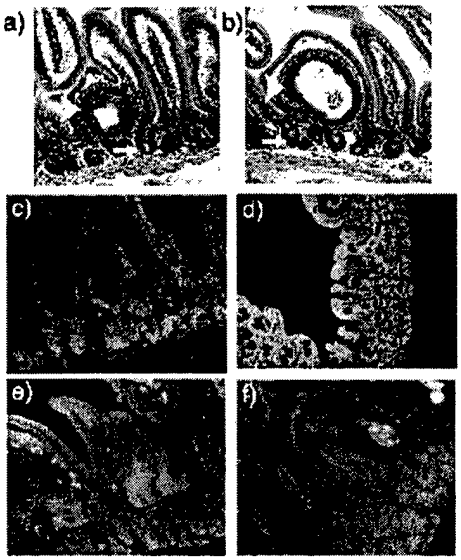

Figure 5. Expression of NKCC1 is maintained in aberrant crypts initiated by the stabilization of β-catenin in the mouse bowels. Mice expressing CreERT2 specifically in the bowels and carrying the $Catnb^{+/lox(ex3)}$ allele were fed with 2 mg tamoxifen for 5 days, and after 2 weeks were sacrificed and examined for early polyps. a&b show newly initiated aberrant crypts on their way to develop into adenomatous polyps (arrow heads), arrows point to normal crypts. c&d show stainings for b-catenin (green) and NKCC1 (orange) in healthy small and large intestine respectively. e&f show the same stainings in aberrant crypts in the small inestine; note that expression of NKCC1 is maintained in the newly arising adenomas.

Stabilization of β-catenin happens to be the primary cause of colon cancer, usually caused by loss of function of its partner, the adenomatous polyposis coli protein. We therefore examined changes in expression of NKCC1 in adenomas caused by the stabilization of β-catenin in the murine bowels. In contrast to PIN, initiation of intestinal polyposis did not coincide with the extinction of NKCC1. Instead, the aberrant crypts continued to express this cell surface marker as they proceeded to traverse the crypt-villus boundary (Figure 5). This observation clearly indicates that extinction of NKCC1 is not simply connected to the stabilization of β-catenin. We then examined expression of NKCC1 in prostate neoplasias caused by expression of a dominant active AKT, or over-expression of the SV40 large T. In both instances expression of NKCC1 continued as the secretory epithelium was transformed (Figure 6). These observations suggest that extinction of NKCC1 is unlikely to be related to generally to loss of epithelial polarization by prostatic eptithelium undergoing neoplastic transformation. We then examined expression of NKCC1 in histologic samples of human PIN. Histology samples of PINs from 11 different patients, all contained lesions that strongly expressed NKCC1, and very rarely did we encounter

examples of extinction of this marker (Figure 7). Finally, we examined the prostate and intestine of NKCC1 deficient mice, to look for evidence suggesting a vital role for this marker in the development and differentiation of secretory epithelia. Both tissues appeared normal. In conclusion our observations suggest that extinction of NKCC1 in β-catenin induced PINs is a unique event linked to the target tissue and its response to β-catenin. NKCC1 appears to be a non-essential component of the secretory epithelia in the prostate (or the bowels), in as far as its loss does not affect the gross appearance or function of these tissues.

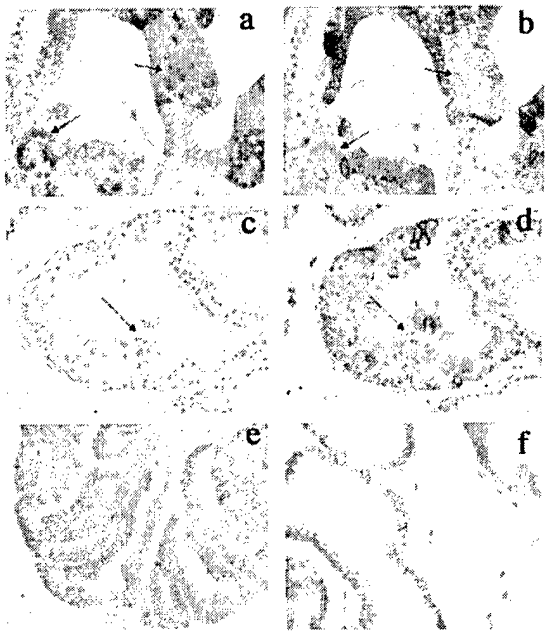

Figure 6. Extinction of NKCC1 occurs only on PINs caused by the stabilization of β-catenin. a) β-catenin and, b) NKCC1 stain respectively of lesions from MMTV-Cre x $Catnb^{+/lox(ex3)}$ mouse prostate; note that the stabilization of β-catenin in "a" coinicides with the loss of NKCC1 in "b". c) β-catenin and, d) NKCC1 staining respectively of PB-AKT mouse prostate; note no change of either staining in the PINs. e & f, NKCC1 staining of prostate lesion from a TRAMP mouse or of control healthy mouse respectively. Arrows point to lesions; note strong staining in both the SV40 induced cancer and in healthy prostate, respectively.

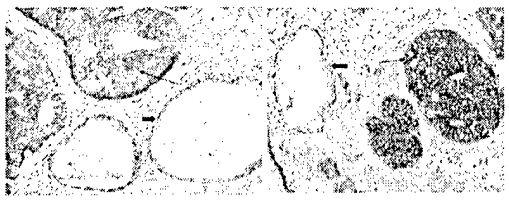

Figure 7. Human PIN express NKCC1. Immunoperoxidase staining for NKCC1, of human histology samples. Thin arrows show the PIN like lesions, thick arrows point to healthy glands.

Key research accomplishments.

- It was demonstrated that stabilization of β-catenin in the prostate secretory epithelia, is responsible for the initiation of PIN.
- It was demonstrated that the extent and progression of neoplasia in the prostate secretory epithelium, its' association with squamous metaplasia, and the occurrence or not of local inflammatory reactions, depend strictly on the promoter that is used to drive expression of Cre and therefore stabilization of β-catenin. This could be related to stage of cellular differentiation that is targeted in each case, by the MMTV-LTR, probasin, or PSA promoter respectively.
- Histologic evidence was provided for the stabilization of β-catenin in human PIN, although this did not seem to be a universal event even amongst PINs from the same prostate.
- Expression of NKCC1 was readily detectable in the majority of cases of human PINs examined, suggesting that its extinction is not a common feature of early stage prostate cancer.
- It was established that extinction of NKCC1 is not a common feature of tumors caused in other tissues by β-catenin, or prostatic neoplasia caused by genetic events other than those that lead to stabilization of β-catenin.

Reportable outcomes.

The first year has focused our attention on the exact experimental systems and approaches that are likely to provide breakthroughs. In the second year, we expect to be able to bring these observations together for publication. We have also initiated experiments with novel animal models that address the PI3kinase/AKT pathway, and anticipate new discoveries leading to publications in the second year.

Conclusions.

Using different transgenic mice we were able to demonstrate that the target of transformation by β-catenin in the prostate is the secretory epithelia. Furthermore, it is the stabiliztion of β-catenin precisely in these cells and their neoplastic differentiation into PIN that leads to the extinction of NKCC1. Thus, the same mutation is responsible for the initiation of adenomatous polyps and colon cancer, but here cell surface expression of NKCC1 is maintained. Also, prostate cancer caused by other genetic events, do not coincide with extinction of NKCC1. Initiation of prostate cancer may involve different genetic events, and this could be one explanation for the different susceptibilities of PINs in different cases to progress to aggressive malignancies. Preliminary data was provided suggesting that the stage of cellular differentiation "hit" by β-catnein can determine the extent and aggression of the PINs. Stabiliztion of β-catenin is also readily detectable in a significant fraction of human PINs examined to date. However, it does not seem to be an exclusive requirement for the cancer, as an equal number of examined human PIN sections did not stain for this protein. It is not yet know how PINs that express high levels of β-catenin fair in comparison to those which don't, with respect to their susceptibility to progress to malignancy. Future work will focus on the comparison of the PSA-Cre and PB-Cre mice to induce prostate specific targeted stabilization of β-catenin, and/or inactivation of PTEN, as well as studies of downstream targets of β-catenin in the prostate physiology and cancer

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BIOGRAPHICAL SKETCH

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EDUCATION

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October 20-29, 2000 Modeling Human Colo-Rectal Cancer in Mice, The Jackson Laboratory, Bar Harbor.
April 3-9, 2002 Mechanisms and Applications of Immune Tolerance, Keystone Symposium, Steamboat, Colorado

ORIGINAL REPORTS:

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Research projects completed or ongoing in the last three years (PI: K. Khazaie)

Ongoing Research Support (PI: K. Khazaie)

Idea Award. May 2002 - May 2005
Department of Defense Breast Cancer Research Program,
Title: Cancer Immunology in an inducible model of breast cancer.
Major goals: To develop an animal model of inducible mammary cancer, and to use this model to study antigen specific immune responses against the mammary gland and mammary tumors.

Idea Award. 2003-2008
Department of Defense Prostate Cancer Research Program,
Title: Initiating events in prostate cancer: The role of somatic activation of β-catenin.
Major goals: To evaluate the stabilization of β-catenin as an initiating event in prostate cancer.

Senior National Research Council Award Jan 2003 – Jan 2005
National Cancer Institute
(Salary Support only)

Completed Research Support
Inter-programmatic Research Award. 2002-2004
Dana Farber Cancer Institute/Harvard Cancer Center
Title: An animal model for investigating immunosurveillance and immunotherapy in prostate cancer.
Major goals: To develop an animal model of prostate cancer based on the prostate specific activation of the APC/β-catenin pathway, and to use this model to study antigen specific immune responses against the prostate.

National Colorectal Cancer Research Alliance. (Award) 2001
Entertainment Industry
Title: An Animal Model for Designing Targeted Immune Intervention in Colon Cancer.
Major goals: To Investigate antigen specific immune responses in the healthy and neoplastic mouse intestine.

Hershey Prostate Cancer/Survivors Walk. (Award) 2001
Beth Israel Hospital
Title: somatic activation of β-catenin reveals a critical event in the initiation of prostate cancer.
Major goals: To Investigate the role of β-catenin in prostate cancer.

Applied for
Claudia Adams Barr Program in Cancer Research. Boston, MA.
Dana Farber Cancer Institute
Title: Mast cells. And orchestration of local inflammatory reactions in colon cancer.
Major goals: To investigate the cross-talk between tumor epithelium and infiltrating mast cells.

RO1 submitted in 2003
Title: Imaging Proteolytic Activity in Colon Cancer
Major goals: Image proteolytic activity in animal models of colon cancer, and apply imaging to detection of tumor status and biological response.

RO1 submitted in Feb 2004
Title: Inflammation in Colon Cancer: A Cause or consequence?
Major goals: Define the role of innate immune response in the initiation of polyposis and in progression of invasive carcinomoma.